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(57) Abstract

The invention provides active, non-naturally occurring mutants of beetle luciferases and DNAs which encode such mutants. A mutant luciferase of the invention differs from the corresponding wild-type luciferase by producing bioluminescence with a wavelength of peak intensity that differs by at least 1 nm from the wavelength of peak intensity of the bioluminescence produced by the wild-type enzyme. The mutant luciferases and DNAs of the invention are employed in various biosensing applications.

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MUTANT LUCIFERASES

TECHNICAL FIELD

This invention generally relates to luciferase enzymes that produce luminescence, like that from fireflies. More particularly, the invention concerns mutant luciferases of beetles. The mutant luciferases of the invention are made by genetic engineering, do not occur in nature, and, in each case, include modifications which cause a change in color in the luminescence that is produced. The luciferases of the invention can be used, like their naturally occurring counterparts, to provide luminescent signals in tests or assays for various substances or phenomena.

BACKGROUND OF THE INVENTION

The use of reporter molecules or labels to qualitatively or quantitatively monitor molecular events is well established. They are found in assays for medical diagnosis, for the detection of toxins and other substances in industrial environments, and for basic and applied research in biology, biomedicine, and biochemistry. Such assays include immunoassays, nucleic acid probe hybridization assays, and assays in which a reporter enzyme or other protein is produced by expression under control of a particular promoter. Reporter molecules, or labels in such assay systems, have included radioactive isotopes, fluorescent agents, enzymes and chemiluminescent agents.

Included in the assay system employing chemiluminescence to monitor or measure events of interest are assays which measure the activity of a bioluminescent enzyme, luciferase.

Light-emitting systems have been known and isolated from many luminescent organisms including bacteria, protozoa, coelenterates, molluscs, fish, millipedes, flies, fungi, worms, crustaceans, and beetles, particularly click beetles of genus *Pyrophorus* and the fireflies of the genera *Photinus*, *Photuris*, and

Luciola. In many of these organisms, enzymes catalyze monooxygenations and utilize the resulting free energy to excite a molecule to a high energy state. Visible light is emitted when the excited molecule spontaneously returns to the ground state. This emitted light is called "bioluminescence." Hereinafter it may also be referred to simply as "luminescence."

The limited occurrence of natural bioluminescence is an advantage of using luciferase enzymes as reporter groups to monitor molecular events. Because natural bioluminescence is so rare, it is unlikely that light production from other biological processes will obscure the activity of a luciferase introduced into a biological system. Therefore, even in a complex environment, light detection will provide a clear indication of luciferase activity.

Luciferases possess additional features which render them particularly useful as reporter molecules for biosensing (using a reporter system to reveal properties of a biological system). Signal transduction in biosensors (sensors which comprise a bilogical component) generally involves a two step process: signal generation through a biological component, and signal transduction and amplification through an electrical component. Signal generation is typically achieved through binding or catalysis. Conversion of these biochemical events into an electrical signal is typically based on electrochemical or caloric detection methods, which are limited by the free energy change of the biochemical reactions. For most reactions this is less than the energy of hydrolysis for two molecules of ATP, or about 70 kJ/mole. However, the luminescence elicited by luciferases carries a much higher energy content. Photons emitted from the reaction catalyzed by firefly luciferase (560 nm) have 214 Kj/einstein. Furthermore, the reaction catalyzed by luciferase is one of the most efficient bioluminescent reactions known, having a

quantum yield of nearly 0.9. This enzyme is therefore an extremely efficient transducer of chemical energy.

Since the earliest studies, beetle luciferases, particularly that from the common North American firefly species *Photinus pyralis*, have served as paradigms for understanding of bioluminescence . The fundamental knowledge and applications of luciferase have been based on a single enzyme, called "firefly luciferase," derived from *Photinus pyralis*. However, there are roughly 1800 species of luminous beetles worldwide. Thus, the luciferase of Photinus pyralis is a single example of a large and diverse group of beetle luciferases. It is known that all beetle luciferases catalyze a reaction of the same substrate, a polyheterocyclic organic acid, D-(-)-2-(6'-hydroxy-2'-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (hereinafter referred to as "luciferin", unless otherwise indicated), which is converted to a high energy molecule. It is likely that the catalyzed reaction entails the same mechanism in each case.

The general scheme involved in the mechanism of beetle bioluminescence appears to be one by which the production of light takes place after the oxidative decarboxylation of the luciferin, through interaction of the oxidized luciferin with the enzyme. The color of the light apparently is determined by the spatial organization of the enzyme's amino acids which interact with the oxidized luciferin.

The luciferase-catalyzed reaction which yields bioluminescence (hereinafter referred to simply as "the luciferase-luciferin reaction") has been described as a two-step process involving luciferin, adenosine triphosphate (ATP), and molecular oxygen. In the initial reaction, the luciferin and ATP react to form luciferyl adenylate with the elimination of inorganic pyrophosphate, as indicated in the following reaction:

$$E + LH_2 + ATP \rightleftharpoons E \cdot LH\text{-}AMP + PP_i$$

where E is the luciferase, LH_2 is luciferin, and PPi is pyrophosphate. The luciferyl adenylate, LH_2-AMP, remains tightly bound to the catalytic site of luciferase. When this form of the enzyme is exposed to molecular oxygen, the enzyme-bound luciferyl adenylate is oxidized to yield oxyluciferin (L=O) in an electronically excited state. The excited oxidized luciferin emits light on returning to the ground state as indicated in the following reaction:

$$E\text{-}LH_2\text{-}AMP + O_2 \Rightarrow (E \cdot L{=}O \cdot AMP)^* + 2H^+ + CO_2$$

$$\downarrow$$

$$E \cdot L = O \cdot AMP + light$$

One quantum of light is emitted for each molecule of luciferin oxidized. The electronically excited state of the oxidized luciferin is a characteristic state of the luciferase-luciferin reaction of a beetle luciferase; the color (and, therefore, the energy) of the light emitted upon return of the oxidized luciferin to the ground state is determined by the enzyme, as evidenced by the fact that various species of beetles having the same luciferin emit differently colored light.

Luciferases have been isolated directly from various sources. The cDNAs encoding luciferases of various beetle species have been reported. (See de Wet et al., Molec. Cell. Biol 7, 725 - 737 (1987); Masuda et al., Gene 77, 265 - 270 (1989); Wood et al., Science 244, 700 - 702 (1989)). With the cDNA encoding a beetle luciferase in hand, it is entirely straightforward for the skilled to prepare large amounts of the luciferase by isolation from bacteria (e.g., E. coli), yeast, mammalian cells in culture, or the like, which have been transformed to express the cDNA. Alternatively, the cDNA, under control of an appropriate promoter and other

signals for controlling expression, can be used in such a cell to provide luciferase, and ultimately bioluminescence catalyzed thereby, as a signal to indicate activity of the promoter. The activity of the promoter may, in turn, reflect another factor that is sought to be monitored, such as the concentration of a substance that induces or represses the activity of the promoter. Various cell-free systems, that have recently become available to make proteins from nucleic acids encoding them, can also be used to make beetle luciferases.

Further, the availability of cDNAS encoding beetle luciferases and the ability to rapidly screen for cDNAS that encode enzymes which catalyze the luciferase-luciferin reaction (see de Wet et al., supra and Wood et al., supra) also allow the skilled to prepare, and obtain in large amounts, other luciferases that retain activity in catalyzing production of bioluminescence through the luciferase-luciferin reaction. These other luciferases can also be prepared, and the cDNAs that encode them can also be used, as indicated in the previous paragraph. In the present disclosure, the term "beetle luciferase" or "luciferase" means an enzyme that is capable of catalyzing the oxidation of luciferin to yield bioluminescence, as outlined above.

The ready availability of cDNAS encoding beetle luciferases makes possible the use of the luciferases as reporters in assays employed to signal, monitor or measure genetic events associated with transcription and translation, by coupling expression of such a cDNA, and consequently production of the enzyme, to such genetic events.

Firefly luciferase has been widely used to detect promoter activity in eucaryotes. Though this enzyme has also been used in procaryotes, the utility of firefly luciferase as genetic reporter in bacteria is not commonly recognized. As genetic reporters, beetle

luciferases are particularly useful since they are monomeric products of a single gene. In addition, no post-translational modifications are required for enzymatic activity, and the enzyme contains no prosthetic groups, bound cofactors, or disulfide bonds. Luminescence from E.coli containing the gene for firefly luciferase can be triggered by adding the substrate luciferin to the growth medium. Luciferin readily penetrates biological membranes and cannot be used as a carbon or nitrogen source by E.coli. The other substrates required for the bioluminescent reaction, oxygen and ATP, are available within living cells. However, measurable variations in luminescence color from luciferases would be needed for systems which utilize two or more different luciferases as reporters (signal geneators).

Clones of different beetle luciferases, particularly of a single genus or species, can be utilized together in bioluminescent reporter systems. Expression in exogenous hosts should differ little between these luciferases because of their close sequence similarity. Thus, in particular, the click beetle luciferases may provide a multiple reporter system that can allow the activity of two or more different promoters to be monitored within a single host, or for different populations of cells to be observed simultaneously. The ability to distinguish each of the luciferases in a mixture, however, is limited by the width of their emissions spectra.

One of the most spectacular examples of luminescence color variation occurs in *Pyrophorus plagiophthalamus*, a large click beetle indigenous to the Caribbean. This beetle has two sets of light organs, a pair on the dorsal surface of the prothorax, and a single organ in a ventral cleft of the abdomen. Four different luciferase clones have been isolated from the ventral organ. The luciferin-luciferase reactions catalyzed by

these enzymes produces light that ranges from green to orange.

Spectral data from the luciferase-luciferin reaction catalyzed by these four luciferases show four overlapping peaks of nearly even spacing, emitting green (peak intensity: 546 nanometers), yellow-green (peak intensity: 560 nanometers), yellow (peak intensity: 578 nanometers) and orange (peak intensity: 593 nanometers) light. The respective proteins are named Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE and Luc*Ppl*OR. Though the wavelengths of peak intensity of the light emitted by these luciferases range over nearly 50 nm, there is still considerable overlap among the spectra, even those with peaks at 546 and 593 nm. Increasing the difference in wavelength of peak intensity would thus be useful to obtain greater measurement precision in systems using two or more luciferases.

The amino acid sequences of the four luciferases from the ventral organ are highly similar. Comparisons of the sequences show them to be 95 to 99% identical.

It would be desirable to enhance the utility of beetle luciferases for use in systems using multiple reporters to effect mutations in luciferase-encoding cDNAs to produce mutant luciferases which, in the luciferase-luciferin reaction, produce light with differences between wavelengths of peak intensity that are greater than those available using currently available luciferases.

Beetle luciferases are particularly suited for producing these mutant luciferases since color variation is a direct result of changes in the amino acid sequence.

Mutant luciferases of fireflies of genus *Luciola* are known in the art. Kajiyama et al., U.S. Patent Nos. 5,219,737 and 5,229,285.

In using luciferase expression in eukaryotic cells for biosensing, it would be desirable to reduce transport of the luciferase to peroxisomes. Sommer et

al., Mol. Biol. Cell 3, 749 - 759 (1992), have described mutations in the three carboxy-terminal amino acids of P. pyralis luciferase that significantly reduce peroxisome-targeting of the enzyme.

The sequences of cDNAs enoding various beetle luciferases, and the amino acid sequences deduced from the cDNA sequences, are known, as indicated in Table I.

Table I

References for cDNA and Amino Acid Sequences of Various Wild-Type Beetle Luciferases

Luciferase	Reference
Luc*Ppl*GR	K. Wood, Ph.D. Dissertation, University of California, San Diego (1989), see also SEQ ID NO:1; Wood *et al.*, Science *244*, 700-702 (1989)
Luc*Ppl*YG	K. Wood, Ph.D. Dissertation, University of California, San Diego (1989); Wood *et al.*, Science *244*, 700-702 (1989)
Luc*Ppl*YE	K. Wood, Ph.D. Dissertation, University of California, San Diego (1989); Wood *et al.*, Science *244*, 700-702 (1989)
Luc*Ppl*OR	K. Wood, Ph.D. Dissertation, University of California, San Diego (1989); Wood *et al.*, Science *244*, 700-702 (1989)
Photinus pyralis	de Wet *et al.*, Mol. Cell. Biol. *7*, 725 - 737 (1987); K. Wood, Ph.D. Dissertation, University of California, San Diego (1989); Wood *et al.*, Science *244*, 700 - 702 (1989)
Luciola cruciata	Kajiyama *et al.*, United States Patent No. 5,229,285; Masuda *et al.*, United States Patent No. 4,968,613
Luciola lateralis	Kajiyama *et al.*, United States Patent No. 5,229,285
Luciola mingrelica	Devine *et al.*, Biochim. et Biophys. Acta *1173*, 121-132(1993)

The cDNA and amino acid sequences of Luc*Ppl*GR, the green-emitting luciferase of the elaterid beetle *Pyrophorus plagiophthalamus*, are shown in SEQ ID NO:1.

SUMMARY OF THE INVENTION

The present invention provides mutant luciferases of beetles and DNAs which encode the mutant luciferases. Preferably, the mutant luciferases produce a light of different color from that of the corresponding wild-type luciferase and preferably this difference in color is such that the wavelength of peak intensity of the luminescence of the mutant differs by at least 1 nm from that of the wild-type enzyme.

The mutant luciferases of the invention differ from the corresponding wild-type enzymes by one or more, but typically fewer than three, amino acid substitutions. The luciferases of the invention may also entail changes in one or more of the three carboxy-terminal amino acids to reduce peroxisome targeting.

In one surprising aspect of the invention, it has been discovered that combining in a single mutant two amino acid substitions, each of which, by itself, occasions a change in color (shift in wavelength of peak intensity) of bioluminescence, causes the mutant to have a shift in wavelength of peak intensity that is greater than either shift caused by the single amino acid substitutions.

cDNAs encoding the mutant luciferases of the invention may be obtained straightforwardly by any standard, site-directed mutagenesis procedure carried out with a cDNA encoding the corresponding wild-type enzyme or another mutant. The mutant luciferases of the invention can be made by standard procedures for expressing the cDNAs which encode them in prokaryotic or eukaryotic cells.

A fuller appreciation of the invention will be gained upon examination of the following detailed description of the invention.

DETAILED DESCRIPTION OF THE INVENTION

In the following description and examples, process steps are carried out and concentrations are measured at room temperature (about 20 °C to 25 °C) and atmospheric pressure unless otherwise specified.

All amino acids referred to in the specification, except the non-enantiomorphic glycine, are L-amino acids unless specified otherwise. An amino acid may be referred to using the one-letter or three-letter designation, as indicated in the following Table II.

Table II

Designations for Amino Acids

Amino Acid	Three-Letter Designation	One-Letter Designation
L-alanine	Ala	A
L-arginine	Arg	R
L-asparagine	Asn	N
L-aspartic acid	Asp	D
L-cysteine	Cys	C
L-glutamic acid	Glu	E
L-glutamine	Gln	Q
glycine	Gly	G
L-histidine	His	H
L-isoleucine	Ile	I
L-leucine	Leu	L
L-lysine	Lys	K
L-methionine	Met	M
L-phenylalanine	Phe	F
L-proline	Pro	P
L-serine	Ser	S
L-threonine	Thr	T
L-tryptophan	Trp	W
L-tyrosine	Tyr	Y
L-valine	Val	V

"X" means any one of the twenty amino acids listed in Table II.

Peptide or polypeptide sequences are written and numbered from the initiating methionine, which is numbered "1," to the carboxy-terminal amino acid.

A substitution at a position in a polypeptide is indicated with [designation for original amino acid]$_{[position\ number]}$[designation for replacing amino acid]. For example, substitution of an alanine at position 100 in a polypeptide with a glutamic acid would be indicated by $Ala_{100}Glu$ or $A_{100}E$. Typically, the substitution will be preceded by a designation for the polypeptide in which the substitution occurs. For example, if the substitution $A_{100}E$ occurs in an hypothetical protein designated "Luck," the substitution would be indicated as Luck-$Ala_{100}Glu$ or Luck-$A_{100}E$. If there is more than one substitution in a polypeptide, the indications of the substitutions are separated by slashes. For example, if the hypothetical protein "Luck" has a substitution of glutamic acid for alanine at position 100 and a substitution of asparagine for lysine at position 150, the polypeptide with the substitutions would be indicated as Luck-$Ala_{100}Glu/Lys_{150}Asn$ or Luck-$A_{100}E/K_{150}N$. To indicate different substitutions at a position in a polypeptide, the designations for the substituting amino acids are separated by commas. For example, if the hypothetical "Luck" has substitutions of glutamic acid, glycine or lysine for alanine at position 100, the designation would be Luck-Ala_{100}/Glu,Gly,Lys or Luck-A_{100}/E,G,K.

The standard, one-letter codes "A," "C," "G," and "T" are used herein for the nucleotides adenylate, cytidylate, guanylate, and thymidylate, respectively. The skilled will understand that, in DNAs, the nucleotides are 2'-deoxyribonucleotide-5'-phosphates (or, at the 5'-end, triphosphates) while, in RNAs, the nucleotides are ribonucleotide-5'-phosphates (or, at the 5'-end, triphosphates) and uridylate (U) occurs in place of T. "N" means any one of the four nucleotides.

Oligonucleotide or polynucleotide sequences are written from the 5'-end to the 3'-end.

The term "mutant luciferase" is used herein to refer to a luciferase which is not naturally occurring

and has an amino acid sequence that differs from those of naturally occurring luciferases.

In one of its aspects, the present invention is a mutant beetle luciferase which produces bioluminescence (i.e., catalyzes the oxidation of luciferin to produce bioluminescence) which has a shift in wavelength of peak intensity of at least 1 nm from the wavelength of peak intensity of the bioluminescence produced by the corresponding wild-type luciferase and has an amino acid sequence that differs from that of the corresponding wild-type luciferase by a substitution at one position or substitutions at two positions; provided that, if there is a substitution at one position, the position corresponds to a position in the amino acid sequence of Luc*Ppl*GR selected from the group consisting of position 214, 215, 223, 224, 232, 236, 237, 238, 242, 244, 245, 247, 248, 282, 283 and 348; provided further that, if there are substitutions at two positions, at least one of the positions corresponds to a position in the amino acid sequence of Luc*Ppl*GR selected from the group consisting of position 214, 215, 223, 224, 232, 236, 237, 238, 242, 244, 245, 247, 248, 282, 283 and 348; and provided that the mutant optionally has a peroxisome-targeting-avoiding sequence at its carboxy-terminus.

Exemplary mutant luciferases of the invention are those of the group consisting of Luc*Ppl*GR-$R_{215}H$, -$R_{215}G$, -$R_{215}T$, -$R_{215}M$, -$R_{215}P$, -$R_{215}A$, -$R_{215}L$, -$R_{223}L$, -$R_{223}Q$, -$R_{223}M$, -$R_{223}H$, -$V_{224}I$, -$V_{224}S$, -$V_{224}F$, -$V_{224}Y$, -$V_{224}L$, -$V_{224}H$, -$V_{224}G$, -$V_{232}E$, -$V_{236}H$, -$V_{236}W$, -$Y_{237}S$, -$Y_{237}C$, -$L_{238}R$, -$L_{238}M$, -$L_{238}Q$, -$L_{238}S$, -$L_{238}D$, -$H_{242}A$, -$F_{244}L$, -$G_{245}S$, -$G_{245}E$, -$S_{247}H$, -$S_{247}T$, -$S_{247}Y$, -$S_{247}F$, -$I_{248}R$, -$I_{248}V$, -$I_{248}F$, -$I_{248}T$, -$I_{248}S$, -$I_{248}N$, -$H_{348}N$, -$H_{348}Q$, -$H_{348}E$, -$H_{348}C$, -$S_{247}F/F_{246}L$, -$S_{247}F/I_{248}C$, -$S_{247}F/I_{248}T$, -$V_{224}F/R_{215}G$, -$V_{224}F/R_{215}T$, -$V_{224}F/R_{215}V$, -$V_{224}F/R_{215}P$, -$V_{224}F/P_{222}S$, -$V_{224}F/Q_{227}E$, -$V_{224}F/L_{238}V$, -$V_{224}F/L_{238}T$, -$V_{224}F/S_{247}G$, -$V_{224}F/S_{247}H$, -$V_{224}F/S_{247}T$, and -$V_{224}F/S_{247}F$.

The following Table III shows spectral properties of these and other exemplary mutant luciferases.

TABLE III

Protein	Spectral Properties		
Luc*Ppl*GR-	peak	shift	width
w.t.	545	0	72
$V_{214}S$	*		
Q	*		
Y	*		
K	*		
L	*		
G	*		
C	*		
E	*		
F	*		
P	*		
H	*		
R	*		
$R_{215}H$	562	17	82
Q	567	22	81
G	576	31	82
T	576	31	84
M	582	37	83
P	588	43	91
S	*		
Y	*		
K	*		
L	*		
C	*		
E	*		
F	*		
$R_{223}L$	549	4	75
Q	549	4	73

*Spectral shift (≥ 2 nm) observed by eye.

TABLE III, cont.

Protein	Spectral Shift		
Luc*Ppl*GR-	peak	shift	width
$R_{223}M$	549	4	75
H	551	4	75
S	*		
Y	*		
K	*		
G	*		
C	*		
E	*		
F	*		
P	*		
$V_{224}I$	546	1	75
S	556	11	70
F	561	16	84
Y	565	20	87
L	578	33	94
H	554	39	69
G	584	39	70
$V_{232}E$	554	9	83
$V_{236}H$	554	9	74
W	554	9	74
$Y_{237}S$	553	8	73
C	554	9	74
$L_{238}R$	544	-1	72
P	555	10	75
Q	557	12	76
S	559	14	73
D	568	23	76
$H_{242}A$	559	14	75

*Spectral shift (≥ 2 nm) observed by eye.

TABLE III, cont.

Protein	Spectral Properties		
Luc*Ppl*GR-	peak	shift	width
$H_{247}S$	561	10	74
$F_{244}L$	555	10	73
$G_{245}S$	558	13	75
E	574	29	79
$S_{247}H$	564	19	72
Y	566	21	79
F	569	24	84
$I_{248}R$	544	-1	72
V	546	1	72
F	548	3	79
T	554	9	75
S	558	13	80
[illegible]	577	32	90
$H_{348}A$	592	47	67
C	563	48	66
N	597	52	67
Q	605	60	72
$V_{214}C/V_{224}A$	559	14	72
$S_{247}F/F_{246}L$	567	22	79
$S_{247}F/I_{248}C$	546	41	84
$S_{247}F/I_{248}T$	596	51	80
$T_{233}A/L_{238}M$	555	10	75
$V_{282}I/I_{283}V$	563	3	73
$V_{224}F/R_{215}G$	584	39	80
$V_{224}F/R_{215}T$	587	42	80
$V_{224}F/R_{215}V$	589	44	80
$V_{224}F/R_{215}P$	597	52	81
$V_{224}F/P_{222}S$	564	3	86

TABLE III, cont.

Protein	Spectral Properties		
Luc*Ppl*GR-	peak	shift	width
$V_{224}F/Q_{277}E$	583	38	85
$V_{224}F/L_{238}V$	575	30	85
$V_{224}F/L_{238}M$	576	31	87
$V_{224}F/S_{247}G$	581	36	84
$V_{224}F/S_{247}H$	581	36	79
$V_{224}F/S_{247}Y$	595	50	88
$V_{224}F/S_{247}F$	597	52	85

"Corresponding positions" in luciferases other than Luc*Ppl*GR can be determined either from alignments at the amino acid level that are already known in the art (see, *e.g.*, Wood *et al.*, Science *244*, 700 - 702 (1989); Devine *et al.*, Biochim. et Biophys. Acta *1173*, 121-132(1993)) or by simply aligning at the amino acid level to maximize alignment of identical or conservatively substituted residues, and keeping in mind in particular that amino acids 195 - 205 in the Luc*Ppl*GR sequence are very highly conserved in all beetle luciferases and that there are no gaps for more than 300 positions after that highly conserved 11-mer in any beetle luciferase aminio acid sequence.

A "peroxisome-targeting-avoiding sequence at its carboxy-terminus" means (1) the three carboxy-terminal amino acids of the corresponding wild-type luciferase are entirely missing from the mutant; or (2) the three carboxy-terminal amino acids of the corresponding wild-type luciferase are replaced with a sequence, of one, two or three amino acids that, in accordance with Sommer et al., supra, will reduce peroxisome-targeting by at least 50 %. If the three carboxy-terminal amino acids of the wild-type luciferase are replaced by a three-amino-acid peroxisome-targeting-avoiding sequence in the mutant, and if the sequence in the mutant is $X_1X_2X_3$, where X_3 is carboxy-terminal, than X_1 is any of the twenty amino acids except A, C, G, H, N, P, Q, T and S, X_2 is any of the twenty amino acids except H, M, N, Q, R, S and K, and X_3 is any of the twenty amino acids except I, M, Y and L. Further, any one or two, or all three, of X_1, X_2, and X_3 could be absent from the mutant (i.e., no amino acid corresponding to the position). The most preferred peroxisome-targeting-avoiding sequence is IAV, where V is at the carboxy-terminus.

In another of its aspects, the invention entails a combination of luciferases, in a cell (eukaryotic or prokaryotic), a solution (free or linked as a reporter to

an antibody, antibody-fragment, nucleic acid probe, or the like), or adhererd to a solid surface, optionally through an antibody, antibody fragment or nucleic acid, and exposed to a solution, provided that at least one of the luciferases is a mutant, both of the luciferases remain active in producing bioluminescence, and the wavelengths of peak intensities of the bioluminescence of the luciferases differ because the amino acid sequences of the luciferases differ at at least one of the positions corresponding to positions 214, 215, 223, 224, 232, 236, 237, 238, 242, 244, 245, 247, 248, 282, 283 and 348 in the amino acid sequence of Luc*Ppl*GR, provided that one or both of the luciferases optionally have peroxisome-targeting-avoiding sequences.

In another of its aspects, the invention entails a DNA molecule, which may be an eukaryotic or prokaryotic expression vector, which comprises a segment which has a sequence which encodes a mutant beetle luciferase of the invention.

Most preferred among the DNAs of the invention are those with segments which encode a preferred mutant luciferase of the invention.

From the description of the invention provided herein, the skilled will recognize many modifications and variations of what has been described that are within the spirit of the invention. It is intended that such modifications and variations also be understood as part of the invetion.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT: Promega Corporation
(ii) TITLE OF INVENTION: Mutant Luciferases
(iii) NUMBER OF SEQUENCES: 1
(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Foley & Lardner
(B) STREET: P. O. Box 1497
(C) CITY: Madison
(D) STATE: Wisconsin
(E) COUNTRY: US
(F) ZIP: 53701-1497
(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/177,081
(B) FILING DATE:3-Jan-1994
(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Scanlon, William J.
(B) REGISTRATION NUMBER: 30136
(C) REFERENCE/DOCKET NUMBER: 19017/148P
(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (608) 258-4284
(B) TELEFAX: (608) 258-4258

(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1632 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA to mRNA
(iii) HYPOTHETICAL: no
(iv) ANTISENSE: no
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATG ATG AAG AGA GAG AAA AAT GTT GTA TAT GGA CCC GAA CCC CTA CAC    48
Met Met Lys Arg Glu Lys Asn Val Val Tyr Gly Pro Glu Pro Leu His
                5                  10                  15

CCC TTG GAA GAC TTA ACA GCA GGA GAA ATG CTC TTC AGG GCC CTT CGA    96
Pro Leu Glu Asp Leu Thr Ala Gly Glu Met Leu Phe Arg Ala Leu Arg
            20                  25                  30

AAA CAT TCT CAT TTA CCG CAG GCT TTA GTA GAT GTG TAT GGT GAA GAA   144
Lys His Ser His Leu Pro Gln Ala Leu Val Asp Val Tyr Gly Glu Glu
        35                  40                  45

TGG ATT TCA TAT AAA GAG TTT TTT GAA ACT ACA TGC CTA CTA GCA CAA   192
Trp Ile Ser Tyr Lys Glu Phe Phe Glu Thr Thr Cys Leu Leu Ala Gln
    50                  55                  60

AGT CTT CAC AAT TGT GGA TAC AAG ATG AGT GAT GTA GTG TCG ATC TGC   240
Ser Leu His Asn Cys Gly Tyr Lys Met Ser Asp Val Val Ser Ile Cys
65                  70                  75                  80

GCG GAG AAC AAT AAA AGA TTT TTT GTT CCC ATT ATT GCA GCT TGG TAT   288
Ala Glu Asn Asn Lys Arg Phe Phe Val Pro Ile Ile Ala Ala Trp Tyr
                85                  90                  95

ATT GGT ATG ATT GTA GCA CCT GTT AAT GAG GGC TAC ATC CCA GAT GAA   336
Ile Gly Met Ile Val Ala Pro Val Asn Glu Gly Tyr Ile Pro Asp Glu
            100                 105                 110

CTC TGT AAG GTC ATG GGT ATA TCG AGA CCA CAA CTA GTT TTT TGT ACA   384
Leu Cys Lys Val Met Gly Ile Ser Arg Pro Gln Leu Val Phe Cys Thr
        115                 120                 125
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AAG AAT ATT CTA AAT AAG GTA TTG GAG GTA CAG AGC AGA ACT GAT TTC   432
Lys Asn Ile Leu Asn Lys Val Leu Glu Val Gln Ser Arg Thr Asp Phe
    130                 135                 140

ATA AAA AGG ATT ATC ATA CTA GAT GCT GTA GAA AAC ATA CAC GGT TGT   480
Ile Lys Arg Ile Ile Ile Leu Asp Ala Val Glu Asn Ile His Gly Cys
145                 150                 155                 160

GAA AGT CTT CCC AAT TTT ATT TCT CGT TAT TCG GAT GGA AAT ATT GCC   528
Glu Ser Leu Pro Asn Phe Ile Ser Arg Tyr Ser Asp Gly Asn Ile Ala
                165                 170                 175

AAC TTC AAA CCT TTA CAT TAC GAT CCT GTT GAA CAA GTG GCA GCT ATC   576
Asn Phe Lys Pro Leu His Tyr Asp Pro Val Glu Gln Val Ala Ala Ile
            180                 185                 190

TTA TGT TCG TCA GGC ACA ACT GGA TTA CCG AAA GGT GTA ATG CAA ACT   624
Leu Cys Ser Ser Gly Thr Thr Gly Leu Pro Lys Gly Val Met Gln Thr
        195                 200                 205

CAT AGA AAT GTT TGT GTC CGA CTT ATA CAT GCT TTA GAC CCC AGG GTA   672
His Arg Asn Val Cys Val Arg Leu Ile His Ala Leu Asp Pro Arg Val
    210                 215                 220

GGA ACG CAA CTT ATT CCT GGT GTG ACA GTC TTA GTA TAT CTG CCT TTT   720
Gly Thr Gln Leu Ile Pro Gly Val Thr Val Leu Val Tyr Leu Pro Phe
225                 230                 235                 240

TTC CAT GCT TTT GGG TTC TCT ATA AAC TTG GGA TAC TTC ATG GTG GGT   768
Phe His Ala Phe Gly Phe Ser Ile Asn Leu Gly Tyr Phe Met Val Gly
                245                 250                 255

CTT CGT GTT ATC ATG TTA AGA CGA TTT GAT CAA GAA GCA TTT CTA AAA   816
Leu Arg Val Ile Met Leu Arg Arg Phe Asp Gln Glu Ala Phe Leu Lys
            260                 265                 270

GCT ATT CAG GAT TAT GAA GTT CGA AGT GTA ATT AAC GTT CCA GCA ATA   864
Ala Ile Gln Asp Tyr Glu Val Arg Ser Val Ile Asn Val Pro Ala Ile
        275                 280                 285

ATA TTG TTC TTA TCG AAA AGT CCT TTG GTT GAC AAA TAC GAT TTA TCA   912
Ile Leu Phe Leu Ser Lys Ser Pro Leu Val Asp Lys Tyr Asp Leu Ser
    290                 295                 300

AGT TTA AGG GAA TTG TGT TGC GGT GCG GCA CCA TTA GCA AAG GAA GTT   960
Ser Leu Arg Glu Leu Cys Cys Gly Ala Ala Pro Leu Ala Lys Glu Val
305                 310                 315                 320

GCT GAG ATT GCA GTA AAA CGA TTA AAC TTG CCA GGA ATT CGC TGT GGA  1008
Ala Glu Ile Ala Val Lys Arg Leu Asn Leu Pro Gly Ile Arg Cys Gly
                325                 330                 335

TTT GGT TTG ACA GAA TCT ACT TCA GCT AAT ATA CAC AGT CTT AGG GAT  1056
Phe Gly Leu Thr Glu Ser Thr Ser Ala Asn Ile His Ser Leu Arg Asp
            340                 345                 350

GAA TTT AAA TCA GGA TCA CTT GGA AGA GTT ACT CCT TTA ATG GCA GCT  1104
Glu Phe Lys Ser Gly Ser Leu Gly Arg Val Thr Pro Leu Met Ala Ala
        355                 360                 365

AAA ATA GCA GAT AGG GAA ACT GGT AAA GCA TTG GGA CCA AAT CAA GTT  1152
Lys Ile Ala Asp Arg Glu Thr Gly Lys Ala Leu Gly Pro Asn Gln Val
    370                 375                 380

GGT GAA TTA TGC ATT AAA GGT CCC ATG GTA TCG AAA GGT TAC GTG AAC  1200
Gly Glu Leu Cys Ile Lys Gly Pro Met Val Ser Lys Gly Tyr Val Asn
385                 390                 395                 400
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AAT GTA GAA GCT ACC AAA GAA GCT ATT GAT GAT GAT GGT TGG CTT CAC  1248
Asn Val Glu Ala Thr Lys Glu Ala Ile Asp Asp Asp Gly Trp Leu His
                405                 410                 415

TCT GGA GAC TTT GGA TAC TAT GAT GAG GAT GAG CAT TTC TAT GTG GTG  1296
Ser Gly Asp Phe Gly Tyr Tyr Asp Glu Asp Glu His Phe Tyr Val Val
            420                 425                 430

GAC CGT TAC AAG GAA TTG ATT AAA TAT AAG GGC TCT CAG GTA GCA CCT  1344
Asp Arg Tyr Lys Glu Leu Ile Lys Tyr Lys Gly Ser Gln Val Ala Pro
        435                 440                 445

GCA GAA CTA GAA GAG ATT TTA TTG AAA AAT CCA TGT ATC AGA GAT GTT  1392
Ala Glu Leu Glu Glu Ile Leu Leu Lys Asn Pro Cys Ile Arg Asp Val
    450                 455                 460

GCT GTG GTT GGT ATT CCT GAT CTA GAA GCT GGA GAA CTG CCA TCT GCG  1440
Ala Val Val Gly Ile Pro Asp Leu Glu Ala Gly Glu Leu Pro Ser Ala
465                 470                 475                 480

TTT GTG GTT ATA CAG CCC GGA AAG GAG ATT ACA GCT AAA GAA GTT TAC  1488
Phe Val Val Ile Gln Pro Gly Lys Glu Ile Thr Ala Lys Glu Val Tyr
                485                 490                 495

GAT TAT CTT GCC GAG AGG GTC TCC CAT ACA AAG TAT TTG CGT GGA GGG  1536
Asp Tyr Leu Ala Glu Arg Val Ser His Thr Lys Tyr Leu Arg Gly Gly
            500                 505                 510

GTT CGA TTC GTT GAT AGC ATA CCA AGG AAT GTT ACA GGT AAA ATT ACA  1584
Val Arg Phe Val Asp Ser Ile Pro Arg Asn Val Thr Gly Lys Ile Thr
        515                 520                 525

AGA AAG GAA CTT CTG AAG CAG TTG CTG GAG AAG AGT TCT AAA CTT TAA  1632
Arg Lys Glu Leu Leu Lys Gln Leu Leu Glu Lys Ser Ser Lys Leu
    530                 535                 540
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CLAIMS

1. A mutant beetle luciferase which has an amino acid sequence that differs from that of the corresponding wild-type luciferase by a substitution at one position or substitutions at two positions; provided that, if there is a substitution at one position, the position corresponds to a position in the amino acid sequence of Luc*Ppl*GR selected from the group consisting of position 214, 215, 223, 224, 232, 236, 237, 238, 242, 244, 245, 247, 248, 282, 283 and 348; and provided further that, if there are substitutions at two positions, at least one of the positions corresponds to a position in the amino acid sequence of Luc*Ppl*GR selected from the group consisting of position 214, 215, 223, 224, 232, 236, 237, 238, 242, 244, 245, 247, 248, 282, 283 and 348.

2. A mutant luciferase according to Claim 1 wherein there is one amino acid substitution.

3. A mutant luciferase according to Claim 1 wherein there are two amino acid substitutions.

4. A mutant luciferase according to Claim 3 wherein each of the amino acid substitutions is at a position corresponding to a position in the amino acid sequence of Luc*Ppl*GR selected from the group consisting of position 214, 215, 223, 224, 232, 236, 237, 238, 242, 244, 245, 247, 248, 282, 283 and 348.

5. A mutant luciferase according to Claim 1 wherein the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, Luc*Ppl*OR, the luciferase of *Photinus pyralis*, the luciferase of *Luciola cruciata*, the luciferase of *Luciola lateralis*, and the luciferase of *Luciola mingrelica*.

6. A mutant luciferase according to Claim 2 wherein the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, Luc*Ppl*OR, the luciferase of *Photinus pyralis*, the luciferase of *Luciola cruciata*, the luciferase of *Luciola lateralis*, and the luciferase of *Luciola mingrelica*.

7. A mutant luciferase according to Claim 3 wherein the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, Luc*Ppl*OR, the luciferase of *Photinus pyralis*, the luciferase of *Luciola cruciata*, the luciferase of *Luciola lateralis*, and the luciferase of *Luciola mingrelica*.

8. A mutant luciferase according to Claim 4 wherein the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, Luc*Ppl*OR, the luciferase of *Photinus pyralis*, the luciferase of *Luciola cruciata*, the luciferase of *Luciola lateralis*, and the luciferase of *Luciola mingrelica*.

9. A mutant luciferase according to Claim 5 wherein the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, and Luc*Ppl*OR.

10. A mutant luciferase according to Claim 6 wherein the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, and Luc*Ppl*OR.

11. A mutant luciferase according to Claim 7 wherein the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, and Luc*Ppl*OR.

12 A mutant luciferase according to Claim 8 wherein the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, and Luc*Ppl*OR.

13. A mutant luciferase of Claim 9 wherein the corresponding wild-type luciferase is Luc*Ppl*GR.

14. A mutant luciferase of Claim 10 wherein the corresponding wild-type luciferase is Luc*Ppl*GR.

15. A mutant luciferase of Claim 11 wherein the corresponding wild-type luciferase is Luc*Ppl*GR.

16. A mutant luciferase of Claim 12 wherein the corresponding wild-type luciferase is Luc*Ppl*GR.

17. A mutant luciferase of Claim 13 wherin the mutant is selected from the group consisting of Luc*Ppl*GR-$R_{215}H$, -$R_{215}G$, -$R_{215}T$, -$R_{215}M$, -$R_{215}P$, -$R_{215}A$, -$R_{215}L$, -$R_{223}L$, -$R_{223}Q$, -$R_{223}M$, -$R_{223}H$, -$V_{224}I$, -$V_{224}S$, -$V_{224}F$, -$V_{224}Y$, -$V_{224}L$, -$V_{224}H$, -$V_{224}G$, -$V_{232}E$, -$V_{236}H$, -$V_{236}W$, -$Y_{237}S$, -$Y_{237}C$, -$L_{238}R$, -$L_{238}M$, -$L_{238}Q$, -$L_{238}S$, -$L_{238}D$, -$H_{242}A$, -$F_{244}L$, -$G_{245}S$, -$G_{245}E$, -$S_{247}H$, -$S_{247}T$, -$S_{247}Y$, -$S_{247}F$, -$I_{248}R$, -$I_{248}V$, -$I_{248}F$, -$I_{248}T$, -$I_{248}S$, -$I_{248}N$, -$H_{348}N$, -$H_{348Q}Q$, -$H_{248}E$, -$H_{348}C$, -$S_{247}F/F_{246}L$, -$S_{247}F/I_{248}C$, -$S_{247}F/I_{248}T$, -$V_{224}F/R_{215}G$, -$V_{224}F/R_{215}T$, -$V_{224}F/R_{215}V$, -$V_{224}F/R_{215}P$, -$V_{224}F/P_{222}S$, -$V_{224}F/Q_{227}E$, -$V_{224}F/L_{238}V$, -$V_{224}F/L_{238}T$, -$V_{224}F/S_{247}G$, -$V_{224}F/S_{247}H$, -$V_{224}F/S_{247}T$, and -$V_{224}F/S_{247}F$.

18. A DNA molecule which comprises a segment which has a sequence which encodes a mutant beetle luciferase which has an amino acid sequence that differs from that of the corresponding wild-type luciferase by a substitution at one position or substitutions at two positions; provided that, if there is a substitution at one position, the position corresponds to a position in the amino acid sequence of Luc*Ppl*GR selected from the group consisting of position 214, 215, 223, 224, 232, 236, 237, 238, 242, 244, 245, 247, 248, 282, 283 and 348; and provided further that, if there are substitutions at two positions, at least one of the positions corresponds to a position in the amino acid sequence of Luc*Ppl*GR selected from the group consisting of position 214, 215, 223, 224, 232, 236, 237, 238, 242, 244, 245, 247, 248, 282, 283 and 348.

19. A DNA molecule according to Claim 18 wherein the encoded mutant luciferase has one amino acid substitution.

20. A DNA molecule according to Claim 18 wherein the encoded mutant luciferase has two amino acid substitutions.

21. A DNA molecule according to Claim 20 wherein, in the encoded mutant luciferase, each of the amino acid substitutions is at a position corresponding to a position in the amino acid sequence of Luc*Ppl*GR selected from the group consisting of position 214, 215, 223, 224, 232, 236, 237, 238, 242, 244, 245, 247, 248, 282, 283 and 348.

22. A DNA molecule according to Claim 18 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, Luc*Ppl*OR, the luciferase of *Photinus pyralis*, the luciferase of *Luciola cruciata*, the luciferase of *Luciola lateralis*, and the luciferase of *Luciola mingrelica*.

23. A DNA molecule according to Claim 19 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, Luc*Ppl*OR, the luciferase of *Photinus pyralis*, the luciferase of *Luciola cruciata*, the luciferase of *Luciola lateralis*, and the luciferase of *Luciola mingrelica*.

24. A DNA molecule according to Claim 20 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, Luc*Ppl*OR, the luciferase of *Photinus pyralis*, the luciferase of *Luciola cruciata*, the luciferase of *Luciola lateralis*, and the luciferase of *Luciola mingrelica*.

25. A DNA molecule according to Claim 21 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, Luc*Ppl*OR, the luciferase of *Photinus pyralis*, the luciferase of *Luciola cruciata*, the luciferase of *Luciola lateralis*, and the luciferase of *Luciola mingrelica*.

26. A DNA molecule according to Claim 22 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, and Luc*Ppl*OR.

27. A mutant luciferase according to Claim 23 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, and Luc*Ppl*OR.

28. A DNA molecule according to Claim 24 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, and Luc*Ppl*OR.

29. A DNA molecule according to Claim 25 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is selected from the group consisting of Luc*Ppl*GR, Luc*Ppl*YG, Luc*Ppl*YE, and Luc*Ppl*OR.

30. A DNA molecule according to Claim 26 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is Luc*Ppl*GR.

31. A DNA molecule according to Claim 27 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is Luc*Ppl*GR.

32. A DNA molecule according to Claim 28 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is Luc*Ppl*GR.

33. A DNA molecule according to Claim 29 wherein, for the encoded amino acid sequence, the corresponding wild-type luciferase is Luc*Ppl*GR.

34. A DNA molecule according to Claim 30 wherin the encoded mutant luciferase is selected from the group consisting of Luc*Ppl*GR-$R_{215}H$, -$R_{215}G$, -$R_{215}T$, -$R_{215}M$, -$R_{215}P$, -$R_{215}A$, -$R_{215}L$, -$R_{223}L$, -$R_{223}Q$, -$R_{223}M$, -$R_{223}H$, -$V_{224}I$, -$V_{224}S$, -$V_{224}F$, -$V_{224}Y$, -$V_{224}L$, -$V_{224}H$, -$V_{224}G$, -$V_{232}E$, -$V_{236}H$, -$V_{236}W$, -$Y_{237}S$, -$Y_{237}C$, -$L_{238}R$, -$L_{238}M$, -$L_{238}Q$, -$L_{238}S$, -$L_{238}D$, -$H_{242}A$, -$F_{244}L$, -$G_{245}S$, -$G_{245}E$, -$S_{247}H$, -$S_{247}T$, -$S_{247}Y$, -$S_{247}F$, -$I_{248}R$, -$I_{248}V$, -$I_{248}F$, -$I_{248}T$, -$I_{248}S$, -$I_{248}N$, -$H_{348}N$, -$H_{348}Q$, -$H_{348}E$, -$H_{348}C$, -$S_{247}F/F_{246}L$, -$S_{247}F/I_{248}C$, -$S_{247}F/I_{248}T$, -$V_{224}F/R_{215}G$, -$V_{224}F/R_{215}T$, -$V_{224}F/R_{215}V$, -$V_{224}F/R_{215}P$, -$V_{224}F/P_{227}S$, -$V_{224}F/Q_{227}E$, -$V_{224}F/L_{238}V$, -$V_{224}F/L_{238}T$, -$V_{224}F/S_{247}G$, -$V_{224}F/S_{247}H$, -$V_{224}F/S_{247}T$, and -$V_{224}F/S_{247}F$.

INTERNATIONAL SEARCH REPORT

Inter. ..ional application No.
PCT/US95/00108

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12N 9/02, 15/53
US CL :435/189; 536/23.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/189, 172.3, 320.1, 252.3, 252.33; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FEBS Letters, Volume 307, No. 2, issued July 1992, G. Sala-Newby et al., "Engineering Firefly Luciferase as an Indicator of Cyclic AMP-Dependent Protein Kinase in Living Cells", pages 241-244, see entire document.	1, 2, 5, 6, 18, 19, 22, 23
X	BIOCHEMICAL JOURNAL, Volume 279, issued November 1991, G. Sala-Newby et al., "Engineering a Bioluminescent Indicator for Cyclic AMP-Dependent Protein Kinase", pages 727-732, see entire document.	1, 2, 5, 6, 18, 19, 22, 23
A	PROTEIN ENGINEERING, Volume 4, No. 6, issued August 1991, N. Kajiyama et al., "Isolation and Characterization of Mutants of Firefly Luciferase Which Produce Different Colors of Light", pages 691-693.	1-34

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 05 APRIL 1995	Date of mailing of the international search report 17APR1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Deborah Frelson for REBECCA PROUTY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Inter. .onal application No.
PCT/US95/00108

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BIOLUMINESCENCE AND CHEMILUMINESCENCE, Volume 5, issued April 1990, K.V. Wood, "Luc Genes: Introduction of Colour Into Bioluminescence Assays", pages 107-114.	1-34
A	JOURNAL OF BIOLUMINESCENCE AND CHEMILUMINESCENCE, Volume 4, issued July 1989, K.V. Wood et al., "Introduction to Beetle Luciferases and Their Applications", pages 289-301.	1-34
A	JOURNAL OF BIOLUMINESCENCE AND CHEMILUMINESCENCE, Volume 4, issued July 1989, K.V. Wood et al., "Bioluminescent Click Beetles Revisited", pages 31-39.	1-34
A	SCIENCE, Volume 244, issued 12 May 1989, K.V. Wood et al., "Complementary DNA Coding Click Betle Luciferases Can Elict Bioluminescence of Different Colors", pages 700-702.	1-34

INTERNATIONAL SEARCH REPORT

Inte: ..onal application No.
PCT/US95/00108

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, BIOSIS, LIFESCI, EMBASE, WPI, BIOTECHDS, CA
search terms: luciferase#, muta? or modif?, gene# or sequence#, beetle# or firefl?, pyrophorus or plagiophthalamus, photinus or luciola